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Prognostic Significance of CD44 and nm23 Expression in Patients With Stage II and Stage IIIA Gastric Carcinoma

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Background and Objectives: Predicting the prognosis in gastric carcinoma patients with intermediate stages is difficult. We investigated the prognostic impacts of CD44 and nm23 expression in a homogeneous group of patients with stage II and IIIA gastric carcinoma who had undergone curative resections.

Methods: A total of 261 paraffin-embedded gastric carcinomas were stained with the monoclonal antibodies CD44 and nm23 using the labeled streptovidin biotin method.

Results: The expression of CD44 and nm23 was detected, respectively, in 31.0% (81/261) and 70.1% (183/261) of all tumors. There was no correlation between CD44 expression and clinicopathological variables. However, nm23 was more frequently expressed in older patients with differentiated adenocarcinoma. A significant difference in 5-year survival rates was found between patients with CD44-positive (43.2%) and CD44-negative tumors (63.4%), ($P = 0.0018$). However, there was no significant difference in 5-year survival rates between patients with nm23-positive (54.7%) and nm23-negative tumors (62.7%) ($P = 0.2734$).

Conclusions: CD44 expression was a significant adverse prognostic factor in gastric carcinoma and may be a predictor of metastatic potential of the primary tumor. By contrast, immunohistochemical detection of nm23 expression was not a predictor of outcome of patients with gastric carcinoma. *J. Surg. Oncol. 1999:71:22–28.*

KEY WORDS: **gastric carcinoma; CD44; nm23; immunohistochemistry; prognosis**

INTRODUCTION

The mortality associated with gastric carcinoma is almost entirely caused by subsequent metastatic disease, and it is not unusual to encounter early recurrence after curative resection of the tumor. In fact, the prognostic assessment of gastric carcinoma still relies mainly on TNM staging, but the wide individual variability in prognosis is observed even at the same stages. The accurate prediction of the metastatic potential of the primary tumor, and hence the probable existence of undetected metastases, would be an important aid to the management of patients with gastric carcinoma. The mechanism by which malignant tumors give rise to distant metastases is still largely unresolved because of its complexity. Among the numerous molecules that are suspected to be involved in the tumor metastasis, CD44 and nm23 are of particular interest. A number of different steps in the complex metastatic process are associated with alterations in the adhesive properties of the tumor cells. CD44 is one of the major molecules mediating adhesion be-

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tween epithelial cells which was originally described as a glycoprotein surface marker of human T lymphocytes defined by monoclonal antibody F10-44-2 [1], and later found to be immunologically identical to the Hermes antigen [2] and the transmembrane receptor for hyaluronate [3,4]. A diverse range of functions and multiple isoforms of CD44 generated by alternative splicing were identified [5,6]. The CD44 expression has been reported to be associated with poor outcomes in non-Hodgkin lymphoma [7,8]. The overexpression of a specific CD44 variant in nonmetastatic tumor cell lines has been reported to lead to metastatic behavior [9].

Homologous CD44 variants are overexpressed in human tumors, suggesting that these molecules also have a role in tumor progression [10] and metastasis [11] in man. Recently, CD44 was reported to be associated with disease recurrence and increased tumor mortality in curatively resected patients with gastric carcinoma [12]. The nm23 molecule is a putative metastasis-suppressor gene which was isolated on the basis of its reduced expression in murine melanoma cell lines of high metastatic potential [13]. Two human nm23 genes known as nm23 H1 and nm23 H2, which are 88% homologous to each other, have been described [14]. The genes are on chromosome 17q22, 18kb apart, and encode for the two polypeptide subunits of a nucleoside diphosphate kinase [15]. The nm23 gene has been shown at the level of both the protein and messenger RNA to have a higher expression in breast tissues of low metastatic potential than in corresponding high metastatic variants [16–18]. On the other hand, there are other tumors for which no association between nm23 expression and metastasis has been observed; e.g., lung and squamous carcinomas of the skin [19,20]. The purpose of this study was to evaluate the prognostic impact of the expression of CD44 and nm23 in a homogeneous group of patients with both stage II and stage IIIA gastric carcinomas.

PATIENTS AND METHODS

Patients

A total of 261 gastric carcinoma patients with stage II (n = 126) and stage IIIA (n = 135) carcinomas were included in this study. All patients had undergone curative resections (i.e., International Union Against Cancer [UICC] R0 resection) with D2 or more lymph node dissection between January 1987 and December 1993. The pathologic staging was performed according to the UICC TNM classification [21]. Mean age of the patients was 56 years (range, 26–77 years). Total gastrectomy was performed in 79 patients (30.3%) and subtotal gastrectomy in 182 patients (69.7%). To eliminate bias due to deaths directly resulting from operation, the study group of 261 patients did not include patients who died within 1 month after surgery. The median follow-up duration for the whole study sample was 63 months (range, 6–124 months). Of the entire group, 141 patients (54%) were still alive, and 120 patients (46%) had died due to recurrent disease or cancer-related causes. To compare the prognostic effect of CD44 and nm23 with other known prognostic parameters—age, sex, tumor location, size, gross and histologic type of tumor, depth of tumor invasion, and lymph node involvement—were evaluated in detail.

Immunohistochemistry

The hematoxylin-eosin stained slides from each patient included in the study were reviewed to confirm the diagnosis of gastric adenocarcinoma and the accuracy of the pathological stage. CD44 staining was performed using mouse monoclonal antibody NCL-CD44 (Novocastra Lab., Newcastle-upon-Tyne, UK) and nm23-H1/NDP-K was detected using mouse monoclonal antibody raised against NDP-kinase A purified from human erythrocytes (NCL-nm23, Novocastra Lab., Newcastle-upon-Tyne, UK). Tissue sections, 6 microns thick, were obtained from the surgical specimens that had been formalin-fixed and embedded in paraffin. The sections were deparaffinized in xylene and rehydrated through a sequence of decreasing concentrations of alcohol down to 70%. They were treated with 3% hydrogen peroxide in methanol for 20 min to remove endogenous peroxidase activity, then washed in Tris-buffered saline (TBS) for 5 min. Nonspecific antibody binding was blocked with phosphate-buffered saline (PBS) containing normal rabbit serum for 20 min. The sections were incubated overnight at 4°C with primary antibodies. After overnight incubation, sections were washed in TBS and then incubated with biotinylated anti-rabbit antiserum, and streptavidin conjugated with horseradish peroxidase complex according to the instructions of the supplier (Dako LSAB kit®, K681, Carpinteria, CA). Sections were again washed in TBS for 10 min, and then the color reaction was developed using AEC (3% 3-amino-9-ethylcarbazole) chromogen substrate (DAKO, K3464, Carpinteria, CA). Sections were counterstained with hematoxylin, cleaned, and mounted.

Omission of the primary antibody was carried out as a negative control, and the positivity was determined by the pathologist, who was blinded to the patients' other prognostic parameters and outcomes.

Scoring

CD44 expression was graded into four easily reproducible subgroups: (a) no detectable expression; (b) questionable or faint expression detected in <5% of tumor cells; (c) strong, heterogeneous, or localized expression in 5% to 75% of tumor cells; and (d) strong and homogeneous expression in >75% of tumor cells. For practical and statistical purposes, subgroups (a) and (b) were classified into a negative group, and subgroups (c) and (d) were classified into a positive group. nm23 ex-

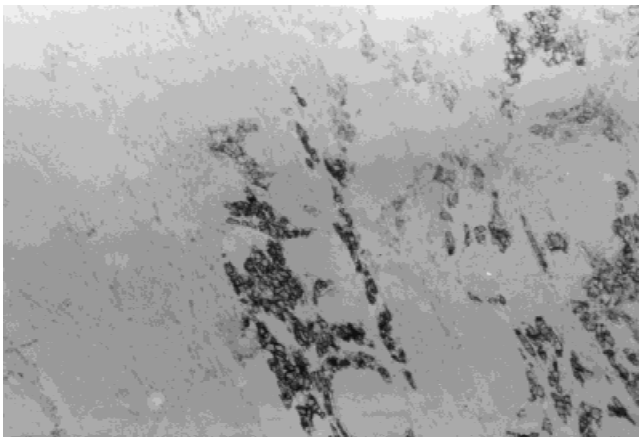

Fig. 1. Immunohistochemical expression of CD44 molecule in the diffuse type of gastric carcinoma. A strong membraneous expression of CD44 molecule is evident.

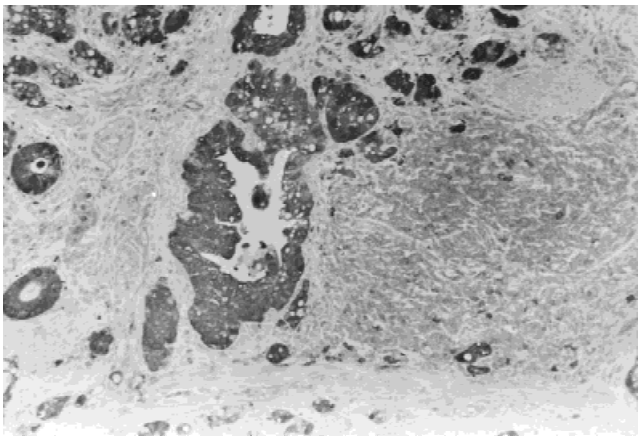

Fig. 2. Immunohistochemical expression of nm23 molecule in the intestinal type of gastric carcinoma. A strong cytoplasmic expression is noted in the most of the gastric carcinoma cells.

pression was scored according to the number of cancer cells stained and the intensity of the reaction in individual cells as weak (stained less intensely than normal mucosa), moderate (stained as intensely as normal mucosa), and strong (stained more intensely than normal mucosa). Samples in which more than 30% of cancer cells were stained with moderate or strong intensity were counted as positive, and samples in which less than 30% of tumor cells were stained with weak or moderate intensity, or in which no reactivity was observed, were counted as negative.

Statistics

Statistical analyses were done with the SPSS 7.5 for Windows program. Frequency tables were analyzed with the χ^2 test. Cumulative survival was estimated by the Kaplan-Meier method, and differences between the patients' groups were tested by the log rank test. The relative importance of risk factors was assessed with Cox's proportional hazard model. Statistical differences with P values <0.05 by two-tailed tests were considered significant.

RESULTS

Immunohistochemical Expression of CD44 and nm23

The expression of CD44 was found in 81 of 261 tumors examined (31.0%), and nm23 was found in 183 of 261 tumors (70.1%). Patterns of immunoreaction are shown and described in detail in Figures 1 and 2. As shown in Table I, the comparison with various clinicopathological features revealed no significant relationship between CD44 overexpression and age, sex, tumor size, location, gross tumor type, histologic differentiation, depth of tumor invasion, and lymph node involvement ($P < 0.05$). No significance was observed when nm23 expression was correlated with sex, tumor size, location, gross tumor type, depth of invasion, and lymph node involvement ($P < 0.05$). There were also no significant correlations between the TNM stagings and the expressions of CD44 ($P = 0.812$) or nm23 protein ($P = 0.931$). A marginally significant association, however, was found between nm23 expression and age ($P = 0.010$), as well as histologic differentiation of the tumor ($P = 0.011$). Thus, tumors in the older age group (50 years) and differentiated tumors were more often associated with nm23 expression than tumors in the younger age group (<50 years) and undifferentiated tumors.

Relationship Between CD44, nm23 Expression and Patients Survival

The 5-year survival rate was 66.4% for patients with stage II and 48.5% for patients with stage IIIA carcinoma ($P = 0.0013$). A significant difference in 5-year survival rates was found between the patients with CD44-positive (43.2%) and CD44-negative tumors (63.4%) ($P = 0.0018$, Fig. 3). On the other hand, there was no significant difference in 5-year survival rates between patients with nm23-positive (54.7%) and nm23-negative tumors (62.7%) ($P = 0.2734$, Fig. 4). In patients with stage II carcinoma, the 5-year survival was 50.0% for those with CD44-positive tumors vs. 74.1% for those with CD44-negative tumors ($P = 0.0063$), and 67.8% for those with nm23-positive tumors vs. 63.2% for those with nm23-negative tumors ($P = 0.5132$). In patients with stage IIIA carcinoma, the 5-year survival rate was 36.6% for those with CD44-positive tumors vs. 53.7% for those with CD44-negative tumors ($P = 0.0831$), and 42.6% for those with nm23-positive tumors vs. 62.4% for those with nm23-negative tumors ($P = 0.0724$).

Multivariate Analysis

We performed a multivariate analysis of survival to test whether CD44 or nm23 adds any information about

TABLE I. Relationships Between Clinicopathological Features and CD44, nm23 Immunoreactivity in Primary Gastric Carcinomas

Clinicopathological features	Number of patients (%)					
	CD44			nm23		
	(−)	(+)	*P* value	(−)	(+)	*P* value
Age (years)			0.668			0.010
<50	49 (27.2)	20 (24.7)		29 (37.2)	40 (21.8)	
≥50	131 (72.8)	61 (75.3)		49 (62.8)	143 (78.2)	
Gender			0.300			0.179
Female	51 (28.3)	18 (22.2)		25 (32.0)	44 (24.0)	
Male	129 (71.7)	63 (77.8)		53 (68.0)	139 (76.0)	
Tumor size (cm)			0.269			0.373
<5	67 (37.2)	36 (44.4)		34 (43.6)	69 (37.7)	
≥5	113 (62.8)	45 (55.6)		44 (56.4)	114 (62.3)	
Tumor location			0.083			0.232
Upper third	26 (14.4)	12 (14.8)		10 (12.8)	28 (15.3)	
Middle third	62 (34.4)	15 (18.5)		23 (29.5)	54 (29.5)	
Lower third	92 (51.1)	54 (66.7)		45 (57.7)	101 (55.2)	
Gross type			0.606			0.113
Borrmann I/II	43 (23.9)	17 (21.0)		13 (16.7)	47 (25.7)	
Borrmann III/IV	137 (76.1)	64 (79.0)		65 (83.3)	136 (74.3)	
Histologic type			0.233			0.011
Differentiated	74 (41.1)	37 (33.3)		21 (26.9)	80 (43.7)	
Undifferentiated	106 (58.9)	54 (66.7)		57 (73.1)	103 (56.3)	
Depth of invasion			0.428			0.912
Serosa (−)	45 (25.0)	24 (29.6)		21 (26.9)	48 (26.2)	
Serosa (+)	135 (75.0)	57 (70.4)		57 (73.1)	135 (73.8)	
Lymph node metastasis			0.573			1.000
(−)	62 (34.4)	25 (30.9)		26 (33.3)	61 (33.3)	
(+)	118 (65.6)	56 (69.1)		52 (66.7)	122 (66.7)	
TNM stage			0.812			0.931
II	86 (47.8)	40 (49.4)		38 (48.7)	88 (48.1)	
IIIA	94 (52.2)	41 (50.6)		40 (51.3)	95 (51.9)	

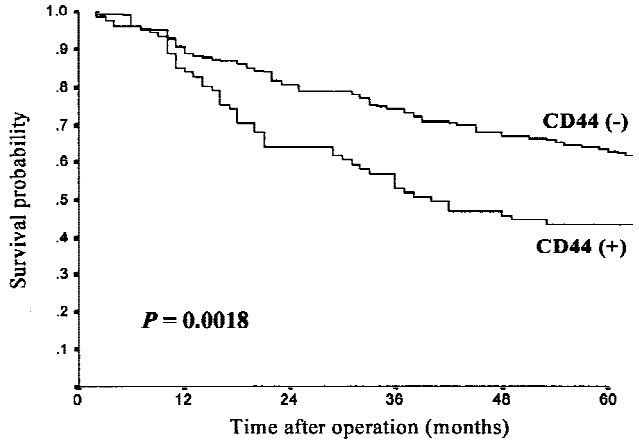

Fig. 3. Survival rates for patients with stage II and IIIA gastric carcinomas in both CD44-positive and CD44-negative groups. There was a significant difference between the two groups ($P = 0.0018$).

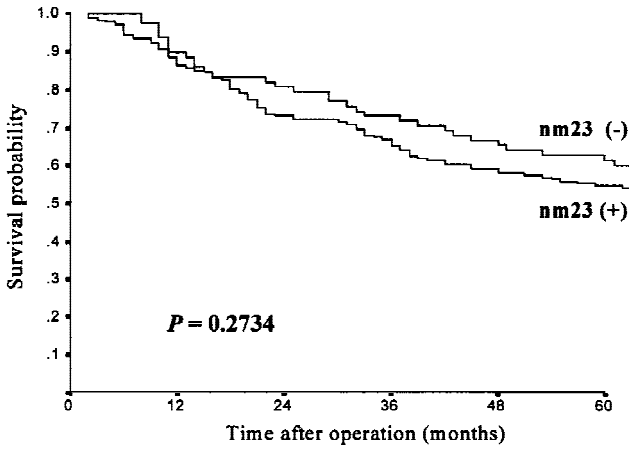

Fig. 4. Survival rates for patients with stage II and IIIA gastric carcinomas in both nm23-positive and nm23-negative groups. There was a no significant difference between the two groups ($P = 0.2734$).

prognosis after standard variables have been allowed for. The results (Table II) indicate that CD44 expression was the most significant risk factor associated with death in stage II and stage IIIA gastric carcinoma (relative risk, 1.78; $P = 0.0051$), followed by lymph node metastasis (relative risk, 1.62; $P = 0.0342$). However, the expression of nm23 showed no significant prognostic effects on survival ($P = 0.1520$).

DISCUSSION

Recently, the prognosis of advanced gastric carcinoma has markedly improved due to the employment of radical

TABLE II. Multivariate Analysis of the Various Prognostic Factors Including CD44 and nm23 Expressions in Stage II and IIIA Gastric Carcinoma Patients

Variable	Coefficient	Standard error	Relative risk	95% Confidence interval	*P* value
CD44	0.58	0.19	1.78	1.23–2.59	0.0051
Node metastasis	0.48	0.23	1.62	1.04–2.52	0.0342
Gross type	0.43	0.25	1.55	0.95–2.53	0.0813
nm23	0.30	0.21	1.35	0.89–2.04	0.1520
Age	0.01	0.01	1.25	0.88–1.86	0.2884
Histologic type	0.14	0.21	1.22	0.81–1.83	0.3291
Tumor location	0.14	0.13	1.15	0.88–1.51	0.2932
Depth of invasion	0.08	0.23	1.09	0.69–1.72	0.7045
Tumor size	0.04	0.21	1.04	0.73–1.55	0.8332
Gender	0.26	0.22	1.02	0.66–1.58	0.9043

surgery and the availability of newer combination therapies. Therefore, theoretically at least, we could expect a complete remission of the disease after a potentially curative operation on patients with advanced gastric carcinoma, especially in stage II and stage IIIA. However, even without evidence of metastatic disease at the time of primary operation, not a few patients in these intermediate stages suffer a progression of their neoplastic disease. To examine this point further in a larger number of specimens, we performed an immunohistochemical analysis of CD44 and nm23 protein, known as metastasis-related genes, in this homogeneous group of gastric carcinoma patients whose differences in biological tumor behavior cannot be explained by traditional prognostic factors.

Several recent immunohistochemical studies have shown that spliced variants containing v6 are overexpressed by aggressive non-Hodgkin lymphoma [22], as well as by human colorectal carcinoma [10]. CD44 variants 4v, 6v, and 9v investigated by immunohistochemical methods were also expressed, but variously, by other types of human malignancies including breast [23], lung [24,25], endometrial [26], ovarian [27], urothelial [28], and gastric carcinoma [29–31]. However, these different reports have led to some controversies, and the relative contribution of each individual CD44 variant in different types of tumor still remains unclear. In gastric carcinoma, little data is available on the clinical significance of the expression of standard form CD44s.

Mayer et al. [12] reported that the use of antibodies that detect all CD44 isoforms to analyze the correlation between CD44 expression and prognosis was possible because, although CD44 isoforms are expressed on most cell types, normal gastric mucosa is CD44 negative; and in benign mucosa, it was expressed only when there was atrophic gastritis associated with a leukocyte infiltrate.

In this study, the overexpression of CD44s was found in 31% of the patients with stage II and stage IIIA gastric carcinoma, and it was close to (35%) or less than (49%) the positive rates of previous reports [31,12]. We were unable to demonstrate a statistically significant correlation between CD44 expression and other traditional prognostic factors such as age, tumor size, location, gross type, differentiation of tumor, depth of tumor invasion, or lymph node metastasis. However, the prognosis of patients with CD44 positive was significantly poorer than that of patients with CD44 negative, and it was more prominent in patients with stage II than patients with stage IIIA. We also demonstrated that CD44 expression provided the most independent prognostic information, followed by lymph node metastasis in multivariate analysis, probably due to excluding the selection bias of patients by different tumor stages.

Although the exact mechanism of CD44 on high malignant potential remains unresolved, our results suggest that immunohistochemical detection of the CD44 protein in routinely fixed gastric carcinoma tissue can be used along with other established parameters to assess prognostic outcome, particularly to identify patients with poor short-term prognosis. Furthermore, this suggests that, in the future, assessment of CD44 expression may guide the clinician in delineating a subset of patients with biologically unfavorable tumors that may profit from postoperative adjuvant therapy.

We could not find any meaningful prognostic relationships for nm23 expression. The significance of nm23 in the metastatic cascade remains a matter of controversy, and its function remains unknown. Judging from the role of NDP kinase, which directly interacts with GTP binding proteins, nm23 probably acts in a signal transduction system of cell-to-cell communication [32,33]. The recent demonstration of the identity of the product of nm23-H1 and NDP kinase A validates the use of the NDP kinase A measurement in evaluating nm23-H1 expression [34]. The antibody NCL-nm23 used in this study recognizes NDP kinase A purified from human erythrocytes. In a number of studies on human malignant tumors such as breast carcinoma [17,18] and colorectal carcinoma [35], a reduced expression of nm23 mRNA or its protein has been correlated with an increased metastatic ability resulting in poorer prognosis. However, there are also several studies reporting a lack of correlation between nm23 expression and metastatic potential in the same types of

carcinoma [36,37]. Furthermore, a high nm23 expression could also be shown to correlate with tumor progression and poor prognosis in colon [38], lung [39,40], and pancreatic carcinoma [41]. In gastric carcinoma, nm23 gene expression was found to be down-regulated in advanced carcinoma, and low nm23 expression was associated with a poor prognosis in the studies of Kodera et al. [42] and Ura et al. [43]. Furthermore, Nakayama et al. [44] were able to demonstrate a reduced immunoreactivity of nm23 in lymph node and liver metastasis when compared with primary gastric carcinoma.

In this study, we evaluated the expression of the nm23 gene at the time of operation in the primary lesions alone. We found that the more histologically differentiated tumors expressed increased levels of nm23—a finding which is consistent with those of Kodera et al. [42] and Ura et al. [43]. However, we could not identify an effect of a down-regulation of the nm23 gene on the prognosis of patients. Although not statistically significant, nm23 positive patients showed a worse survival rate than nm23 negative patients, despite its putative function as a metastasis suppressor gene. Similar results have been reported recently by Müller et al. [45] and Kröning et al. [46]. These apparently contradictory results may indicate that nm23 can function as a suppressor gene in some types of cancer, but can be associated with tumor aggressiveness in others. The variable results obtained by immunohistochemical staining may be caused by multiple factors, such as the number of patients, histologic type of tumors, tumor stages, periods of follow-up, type of antibody used, the methods of fixation, detection, and scoring. Therefore, the precise role of CD44 and nm23 in gastric carcinoma remains to be established; and clearly, further studies are required comparing the results using different antibodies and using reagents specific for the CD44 or NDP-K/nm23 subtypes.

CONCLUSIONS

The data on 261 patients with stage II and stage IIIA gastric carcinoma were analyzed with respect to clinicopathological features and survival rates according to the immunohistochemical expression of CD44 and nm23. The findings of this study support the hypothesis that the overexpression of CD44 products is linked to an increased malignant potential of gastric carcinoma, and thus, that patients with CD44-positive carcinoma have a shortened survival. On the other hand, the immunohistochemical detection of nm23 expression is currently not a predictor of outcome in patients with gastric carcinoma or in identifying subgroups of patients who may be at a higher risk.

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